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Production of Anti-Fungal Substance(s) in the "Sekiguchi Lesion" Induced by *Pyricularia oryzae* Infection on Rice cv. Sekiguchi-asahi*

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Abstract The anti-fungal substance(s) were isolated from the "Sekiguchi lesion" formed on rice cv. Sekiguchi-asahi by *Pyricularia oryzae* infection. These substance(s) were partially purified by silica gel column chromatography. Although spore germination of *P. oryzae* was completely inhibited in solution of the anti-fungal substance(s) at concentrations of 0.1-2.5 mg/ml, but that of *Bipolaris oryzae* was not. When a spore suspension of *P. oryzae* was inoculated to inner surface of rice leaf-sheaths which had been pre-treated with the solution of 0.5-2.5 mg/ml of the anti-fungal substance(s) for 24 hr, penetration and hyphal growth were significantly inhibited. The same phenomenon was also observed in *B. oryzae* infection at the concentration of 0.5 and 1.0 mg/ml of the anti-fungal substance(s). However, phenomena associated with anti-fungal substance-induced resistance such as inhibition of penetration and hyphal growth disappeared within 24 hr after removal of the anti-fungal substance(s). Further, inhibitive effect was not observed in thermal-treated sheaths before the treatment of the anti-fungal substance(s). These results suggest that the anti-fungal substance(s) isolated from the "Sekiguchi lesion" have two biological activities; one is direct effect to the pathogens such as inhibition of spore germination and another is indirect effect to the plants such as induction of disease resistance.

Key words: Anti-fungal substance; *Pyricularia oryzae*; rice blast.

Introduction

It is well known that there are many fungal diseases in rice plants. Among these, rice blast disease caused by *Pyricularia oryzae* Cavara (teleomorph, *Magnaporthe grisea* (Hebert) Barr) is a major disease. Rice cv. Sekiguchi-asahi, which showed unique response to the infection of *P. oryzae* or *Bipolaris oryzae* (Breda de Haan) Shoemaker or to chemical treatments, was found as a mutant of cv. Asahi (Sekiguchi and Furuta, 1965). When this cultivar was infected with *P. oryzae,* large orange to orange brown lesions were formed on the leaves. This unique lesion was named "Sekiguchi lesion" (Kiyosawa, 1970). According to genetic studies (Kiyosawa, 1970; Marchetti *et al.*, 1983), the "Sekiguchi lesion" was controlled by a single recessive gene, *sl,* and rice cv. Sekiguchi-asahi has a blast resistance gene *Pi-a,* too. In a previous report, we demonstrated that the compatible race of *P oryzae* induced typical blast lesions besides the "Sekiguchi lesions", although

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the incompatible races induced only the "Sekiguchi lesions" on cv. Sekiguchi-asahi. Further, hyphal growth of the incompatible races was strongly inhibited in the sheath cells. No sporulation of *P. oryzae* was observed on the "Sekiguchi lesion" produced by the compatible or incompatible race (Arase *et al.*, 1988). These observations suggested that the anti-fungal substance(s) were produced in the "Sekiguchi lesions".

In this paper, we report biological activities of anti-fungal substance(s) produced in the "Sekiguchi lesion".

Materials and Methods

Plant: Rice plants (*Oryza sativa* L., cvs. Mohkotou and Sekiguchi-asahi) were used. Seedlings were planted the soil (Sun soil S®, Nagata Co.) containing the fertilizer in seedling cases, and then grown in a greenhouse.

Fungus and sporulation: Isolates Naga 69-150 (race 007) and SH 85-125 (race 001) of *P. oryzae* and *B. oryzae* were used. Spores of *P. oryzae* were synchronously formed by the method reported previously (Arase *et al.*, 1990). Spores of *B. oryzae* were obtained by growing the fungus on PSA medium for 14-20 days at 26 ℃.

Inoculation: Rice cv. Sekiguchi-asahi at 4-5.5 leaf-stages was inoculated with a spore suspension (1×10^5 spores/ml) of *P. oryzae* (isolate SH 85-125). Inoculated plants were kept in the moist chambers at the room temperature for 24 hr and then kept in the greenhouse. The "Sekiguchi lesions" were collected and then used for extraction and isolation of anti-fungal substance(s).

Extraction of anti-fungal substances: "Sekiguchi lesions"(5 g fresh weight) were extracted with 80 % methanol (300 ml) at room temperature under dark. After 7 days, methanol-soluble fraction was evaporated at 35 ℃ under the reduced pressure. Aqueous layer was used for isolation of anti-fungal substance(s). Methanol-soluble fraction from the "Sekiguchi lesions" was extracted with benzene and then ethyl acetate, successively. Benzene and ethyl acetate extracts prepared from the "Sekiguchi lesion" of 1 g fresh weight were dissolved in 4 ml of distilled water, respectively. These two fractions were used for the bioassay of the anti-fungal activity.

Chromatography: Silica gel column chromatography (Wakogel C200, Wako Co.) was conducted by successive elution with chloroform and methanol, increasing the concentration of latter stepwise.

Bioassay for anti-fungal activity: Spores from isolate Naga 69-150 of *P. oryzae* and *B. oryzae* were suspended in each test solutions. Final spore concentration in each test solution was adjusted to 3×10^4 spores/ml. Fifty μl of a spore suspension was dropped on the hole grass slide, and then kept in the moist chamber at 26 ℃. After 12 or 24 hr, spore germination was investigated.

Effect of anti-fungal substance(s) on pathogenesis: The anti-fungal substance solutions at different concentrations (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 mg/ml) were treated to inner surface of rice leaf-sheaths of cv. Mohkotou at 26 ℃ for 24 hr. The treated sheaths were rinsed with distilled water and then

immediately inoculated with a spore suspension (1×10^4 spores/ml) of isolate Naga 69-150 of *P. oryzae* and *B. oryzae*. After 36 hr, infection behaviors were investigated by Takahashi's method (1951).

Results

Anti-fungal activity of methanol extract from the "Sekiguchi lesion"

Spore germination of *P. oryzae* was significantly inhibited in solution containing the benzene extract. However, in solution containing the ethyl acetate extract, spores did not germinate (Table 1).

Table 1. Fractionation of anti-fungal substance(s) from methanol-soluble fraction of the "Sekiguchi lesions".

Fraction	Spore germination (%)
Distilled water	91.5
Benzen extract	0.0
Ethyl acetate extract	69.0

The "Sekiguchi lesions" were extracted with 80 % methanol for 7 days. After removal of methanol, aqueous layer was extracted with benzene and then ethyl acetate, sucessively. A spore suspension of *P. oryzae* in the solution of benzene and ethyl acetate extracts was incubated for 24 hr at 26 ℃.

Silica gel column chromatography was used for the purification of the anti-fungal substance(s) from benzene extract using chloroform-methanol system (100 : 0, 90 : 10, 50 : 50 and 0 : 100, v/v). Active component(s) were eluted by chloroform (Table 2).

Table 2. Effect on spore germination of each fraction eluted from silica gel column.

Fraction	Spore germination (%)
Distilled water (control)	91.5
Chloroform	12.5
Chloroform : methanol (9 : 1, v/v)	84.0
〃 (1 : 1, v/v)	94.0
Methanol	92.0

Spore germination was investigated 24 hr after incubation.

When spores of *P. oryzae* were incubated in solutions at different concentrations (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 mg/ml) of the chloroform-eluted fraction for 12 or 24 hr, germination was significantly inhibited at concentration of more than 0.1 mg/ml. There is no difference in germination rate between 12 and 24 hr-incubations (Fig. 1).

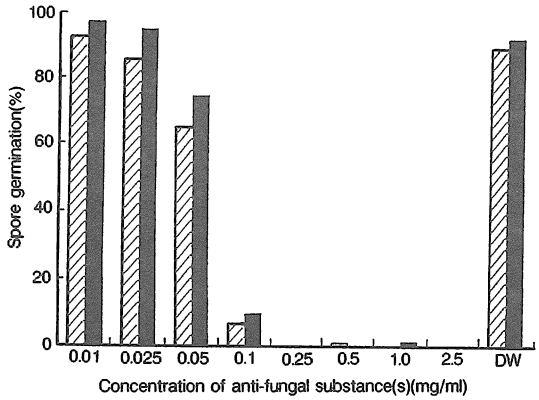

Fig. 1. Effect of anti-fungal substance(s) on spore germination of *P. oryzae*. Spores from isolate Naga 69-150 of *P. oryzae* were incubated in solutions at concentrations of 0 (DW), 0.01, 0.025, 0.1, 0.25, 0.5, 1.0 and 2.5 mg/ml of anti-fungal substance(s). Spore germination was investigated 12(▨) and 24 hr (■) after incubation.

Anti-fungal activity to B. oryzae

When spores of *B. oryzae* were incubated in solutions at different concentrations (0, 0.25, 0.5, 1.0 and 2.5 mg/ml) of the chroloform-eluted fraction for 12 or 24 hr, no inhibition of germination was observed in all concentrations tested (Table 3).

Table 3. Effect of anti-fungal substance(s) on spore germination of *B. oryzae*.

Concentration (mg/ml)	Spore germination (%)	
	12hr	24hr
0	97.8	97.0
0.25	90.8	91.5
0.5	95.0	97.5
1.0	97.0	98.8
2.5	97.5	98.8

A spore suspension of *B. oryzae* in the solution of anti-fungal substance(s) was incubated at 26 ℃. After 12 or 24 hr, spore germination was investigated.

Effect of anti-fungal substance(s) on pathogenesis of P. oryzae and B. oryzae

Penetration and hyphal growth of *P. oryzae* in rice leaf-sheaths of cv. Mohkotou were inhibited with increasing concentrations of the anti-fungal substance(s), and particularly the inhibition at concentrations of 0.5-2.5 mg/ml of the anti-fungal substance(s) was significant (Fig. 2). The inhibition of penetration was also observed at concentrations of 0.5 mg/ml and more in *B. oryzae* (Table 4).

Table 4. Effect of pre-treatment of anti-fungal substance(s) on penetration of *B. oryzae* in rice leaf-sheaths.

Concentration (mg/ml)	Penetration (%)
0	74.6
0.5	1.3
1.0	2.0

The anti-fungal substance(s) were treated to rice leaf-sheaths of cv. Mohkotou at 26 ℃. After 36 hr, penetration was investigated.

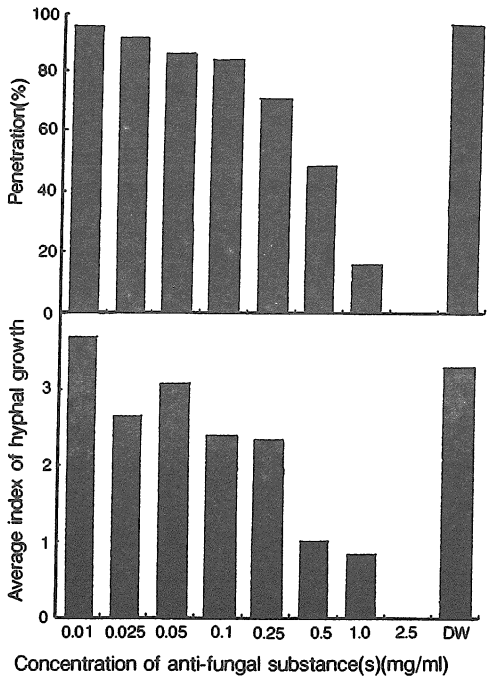

Fig. 2. Effect of pre-treatment of anti-fungal substance(s) on penetration and hyphal growth of *P. oryzae* in rice leaf-sheath cells. Leaf-sheaths which had been treated with solutions at concentrations of 0 (DW), 0.01, 0.025, 0.1, 0.25, 0.5, 1.0 and 2.5 mg/ml of anti-fungal substance(s) for 24 hr at 26 ℃ were inoculated with *P. oryzae* after removal of anti-fungal substance(s). After 36 hr, penetration and hyphal growth of *P. oryzae* were investigated by Takahashi's method (1951).

However, when the sheaths which had been treated with distilled water for 24 hr after removal of the anti-fungal substance(s) from the sheaths were inoculated with *P. oryzae,* inhibition of penetration and hyphal growth was not significant (Fig. 3).

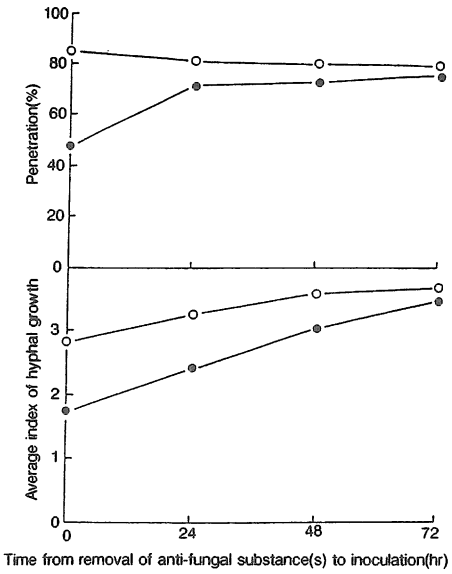

Fig. 3. Duration of effective inhibition of *P. oryzae* infection in rice leaf-sheaths by the anti-fungal substance(s). Leaf-sheaths which had been treated with anti-fungal substance(s) at concentration of 0.5 mg/ml (−●−) or with distilled water (−○−) were rinsed with distilled water and then inoculated with *P. oryzae* 0, 24, 48 and 72 hr after rinse with distilled water. Penetration and hyphal growth of *P. oryzae* were investgated by Takahashi's method (1951).

Effect of heat shock before anti-fungal substance treatment on pathogenesis of P. oryzae and B. oryzae

To demonstrate whether inhibition of fungal penetration and hyphal growth are due to direct or indirect action by the anti-fungal substance(s), anti-fungal substance solution at concentration of 0.5 mg/ml was treated to leaf-sheaths which had been heat-shocked at 55 ℃ for 10 sec. In heat-shocked sheaths, inhibition of penetra-

Table 5. Effect of heat shock treatment of the leaf-sheath on the biological activity of anti-fungal substance(s) in the infection behavior of *P. oryzae*.

Heat treatment	Anti-fungal substance(s)	Penetration(%)	Hyphal growth
No	Distilled water	58.2	2.7
Yes	Distilled water	65.5	3.2
No	0.5 mg/ml	49.0	1.0
Yes	0.5 mg/ml	73.3	3.0

Rice leaf-sheaths of cv. Mohkotou which had been heat-shocked at 55 ℃ for 10 sec were treated with distilled water or anti-fungal substance(s) at concentration of 0.5 mg/ml for 24 hr. After removal of each solution, a spore suspension (1×10^4 spores/ml) of *P. oryzae* was inoculated to leaf-sheaths at 26 ℃. After 36 hr, penetration and hyphal growth were investigated by Takahashi's method (1951).

tion and hyphal growth was not observed (Table 5).

Discussion

This study indicated that the anti-fungal substance(s) were produced in the "Sekiguchi lesion" formed by *P. oryzae*. Such anti-fungal activity was not observed in healthy leaves of cv. Sekiguchi-asahi. This observation suggests the possibility that these substance(s) are phytoalexins of rice plants. In previous studies, Momilactone A and B (Cartwright *et al.*, 1977), Oryzalexin A-D (Akatsuka *et al.*, 1983, 1985; Sekido *et al.*, 1986) and Sakuranetin (Kodama *et al.*, 1992) were isolated from blast infected rice leaves as phytoalexins. However, chemical structure of our substance(s) was unknown at this stage.

Biological activities of the anti-fungal substance(s) were different between *P. oryzae* and *B. oryzae*. Although spore germination of *P. oryzae* was significantly inhibited in anti-fungal substance solution at more than 0.5 mg/ml, that of *B. oryzae* was not. The anti-fungal substance(s) having same activity were also isolated from the "Sekiguchi lesions" formed naturally. From these lesions, *Bipolaris* spp. were more frequently isolated as compared with *P. oryzae* (Kurihisa and Arase, 1986). These results indicate that the anti-fungal activity is blast fungus-specific. Such blast-specific activity has not been reported in the phytoalexins cited above. Our results fit in a previous result that sporulation of *B. oryzae* is very well on the "Sekiguchi lesion", contrasting with no sporulation of *P. oryzae*.

Penetration and hyphal growth of *P. oryzae* and *B. oryzae* were inhibited in the sheaths which had been pre-treated with the anti-fungal substance(s). However, such inhibition in hyphal growth of pathogens was not observed in thermal-treated sheaths before the treatment of the anti-fungal substance(s). These results indicate that the inhibition of infection was not due to direct effect of the anti-fungal substance(s) to pathogens, but to indirect effect such as induction of resistance to rice plants.

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